# Association of plasma CD163 concentration with *de novo*-onset chronic graft-versus-host disease

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**Running head:** Association of plasma CD163 with chronic GVHD

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**Highlights**

- We aimed to identify prognostic plasma proteins associated with onset of chronic GVHD.
- High-throughput mass spectrometry was used to screen pooled plasma samples.
- Enzyme-linked immunosorbent assays were used to test individual samples.
- Higher CD163 concentrations at day 80 were associated with *de novo*-onset chronic GVHD.
- Monocyte or macrophage activation may contribute to the pathogenesis of chronic GVHD.

**Abstract**

Chronic graft-versus-host disease (cGVHD) is the leading cause of long-term morbidity and mortality after allogeneic hematopoietic cell transplantation. In order to identify prognostic plasma proteins associated with *de novo* or quiescent-onset cGVHD, we performed a discovery and validation proteomic study. The total study cohort included 167 consecutive patients who had no clinical evidence of GVHD under minimum glucocorticoid administration, and had available plasma samples obtained at 80±14 days after transplantation. We first used high-throughput mass spectrometry to screen pooled plasma using 20 cases with subsequent cGVHD and 20 controls without it, and identified 20 candidate proteins. We then measured 12 of the 20 candidates by enzyme-linked immunosorbent assays on the same individual samples and identified 4 proteins for further verification (LGALS3BP, CD5L, CD163 and TXN for *de novo* onset, and LGALS3BP and CD5L for quiescent onset). The verification cohort included 127 remaining patients. The cumulative incidence of *de novo*-onset cGVHD was higher in patients with higher plasma soluble CD163 concentrations at day 80 than those with lower concentrations (75% versus 40%, $P$=0.018). The cumulative incidence of *de novo* or quiescent-onset cGVHD did not differ statistically according to concentrations of the three other proteins at day 80. CD163 is a macrophage scavenger receptor and is elevated in oxidative conditions. These results suggest that monocyte or macrophage activation or increased oxidative stress may contribute to the pathogenesis of cGVHD.

## Introduction

Chronic graft-versus-host disease (cGVHD) occurs in approximately 30-50% of patients after allogeneic hematopoietic cell transplantation (HCT) and is the leading cause of late morbidity and mortality.[1] The disease usually occurs beyond 80 days after HCT, and the median onset is 5 months after HCT.[2] This complication is thought to occur because the donor immune system recognizes recipient tissues, causing inflammation and fibrosis.[3, 4]

High-throughput mass spectrometry is a powerful, comprehensive and reduced-bias approach to identify proteomic profiles in many diseases.[5] This approach has been very successful in identifying plasma biomarker proteins that correlate with activity and treatment response of acute GVHD[6-11] and diagnosis and severity of chronic GVHD.[12, 13] To date, prognostic plasma biomarker proteins associated with subsequent onset of chronic GVHD have not been examined by mass spectrometry. Furthermore, few studies have considered the influence of glucocorticoid dosing when the sample was obtained, despite the large influence of glucocorticoids on gene expression and the concentrations of plasma proteins.[12, 14] We hypothesized that comparing plasma proteomic profiles before the onset of chronic GVHD between patients with and without subsequent chronic GVHD could identify prognostic proteins associated with the development of chronic GVHD.

## Methods

### Study design

In order to identify prognostic plasma proteins associated with the subsequent *de novo* or quiescent-onset cGVHD, we performed a discovery and validation proteomic study using high-throughput mass spectrometry. The study was carried out in 3 phases: (i) an initial discovery phase testing pooled plasma samples by mass spectrometry; (ii) confirmation of the candidate

proteins in individual samples of the discovery cohort by using enzyme-linked immunosorbent assays (ELISA); and (iii) verification of candidate proteins in the remaining patients.

**Patients and sample collection**

Patients gave written consent allowing blood sample collection and the use of medical records for research in accordance with the Declaration of Helsinki. The institutional review board of the Fred Hutchinson Cancer Research Center approved the study. Patients were recruited before transplantation, and blood samples were prospectively collected and cryopreserved at 80±14 days after transplantation. Plasma was collected in EDTA and aliquoted in 0.5mL tubes and stored at -80°C within 2 hours of phlebotomy. Acute GVHD was diagnosed and graded according to the previously described criteria.[15, 16] Chronic GVHD was diagnosed by the National Institutes of Health consensus criteria.[17]

The total study cohort included 167 consecutive relapse-free patients who met all of the following criteria: (1) allogeneic hematopoietic cell transplantation (HCT) at the Fred Hutchinson Cancer Research Center/Seattle Cancer Care Alliance between April 2003 and December 2011, (2) available plasma samples at day 80±14 days after HCT, (3) no prior cGVHD at the time of sample collection, (4) no active GVHD at the time of sample collection, and (5) prednisone-equivalent steroid doses ≤5 mg daily at sample collection. Patients were eligible regardless of the indication for HCT, conditioning regimen, graft source, donor relationship and HLA-matching between the donor and recipient.

**Discovery and verification cohorts**

Patients who subsequently developed systemically treated cGVHD were declared cases, while patients without subsequent cGVHD were declared controls. The discovery cohort included two pools from 40 patients selected from the 167 patients described above: one pool of 20 patients with prior acute GVHD and another pool of 20 without prior acute GVHD. Two independent

intact protein analyses (IPA) were done with pooled plasma samples according to presence or absence of prior acute GVHD. Among 20 patients with no prior acute GVHD (IPA1), 10 patients were cases and 10 were controls. Likewise, among 20 patients with prior acute GVHD (IPA2), 10 were cases and 10 were controls. Cases and controls were matched for recipient gender (Table 1). The verification cohort included the 127 remaining patients.

**Mass spectrometric analysis**

Samples were depleted of the 6 most abundant proteins (albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin), pooled and labeled with light acrylamide (cases: subsequent cGVHD-positive) or with a heavy 1,2,3-$^{13}$C-acrylamide (controls: subsequent cGVHD-negative).[5, 18] The pool of cases and the pool of controls were mixed together before further processing and IPA analysis. Proteins were separated by an automated online 2D-HPLC system controlled by Workstation Class-VP 7.4 (Shimadzu Corporation).[5, 18] Separation consisted of anion exchange chromatography followed by reverse-phase chromatography. In-solution tryptic digestion was conducted with lyophilized aliquots from the reverse-phase (second dimension) fractionation step.[13, 23] Aliquots were subjected to tandem mass spectrometry shotgun analysis using an LTQ-Orbitrap (Thermo) mass spectrometer coupled with a NanoLC-1D (Eksigent) on a 2-hour gradient.[13, 23]

**Data processing**

Raw machine output files from all mass spectrometry runs were converted to mzXML files and searched with X!Tandem[19] configured with the k-score scoring algorithm[20] against the Uniprot database. Peptide identifications were assigned probability by PeptideProphet,[21] with a model built on all sample fractions together. As a conservative quality filter, only those identifications with an individual identification probability of 0.95 or higher were retained. The Q3 algorithm for labeled quantitation[22] was applied to estimate quantitative ratios for all cysteine-containing

peptides, with a correction for the overlap between light and heavy isotopic peaks. The Qurate algorithm[23] within the msInspect platform[24] was applied in an automated fashion to locate and remove peptide quantitative events likely to be incorrect due to apparent coeluting peptides, poor isotopic peak distribution, or missing isotopic peaks.

The remaining peptide identifications were provided to ProteinProphet[25] for protein inference. Since only high-quality peptide identifications were used, protein probability assignments were ignored, except to exclude proteins whose evidence was superseded by another protein that explained more of the available peptide evidence. Peptide quantitative ratios assigned by Q3 were combined for each protein, and protein ratios were calculated using the geometric mean of all remaining retained quantitative ratios.

For proteins determined to be of interest due to extreme protein-level quantitative ratios, Qurate was used again, in a manual analysis mode. Peptide quantitative events for these proteins were evaluated on an individual basis, without respect to which protein they represented, and events that visually appeared questionable or incorrect were removed from the analysis. Proteins were selected for further evaluation only if they were supported by quantitative events that passed both automated and manual inspection. The 6 depleted proteins and hemoglobin were excluded from candidate proteins.

**ELISA**

Antibody pairs were purchased as follows: IgM (GENWAY, San Diego, CA), lectin galactoside-binding soluble 3 binding protein (eBioScience, San Diego, CA), gastric inhibitory polypeptide (Millipore, Billerica, MA), CD5 molecule-like (CircuLex, Nagano, Japan), latent transforming growth factor beta binding protein 2 and haptoglobin-related protein (USCN Life Science, Hubei, China), CD163 (R&D Systems, Minneapolis, MN), cartilage acidic protein 1 and PDZ and LIM domain 1 (My BioSource, San Diego, CA), trefoil factor 2 (BioVendor, Asheville, NC), thioredoxin (Immuno-Biological Laboratories, Gunma, Japan), and heat shock protein 90kDa

beta (Grp94), member 1 (Abcam, Cambridge, MA). ELISA assays were performed according to manufacturer's protocols.

**Statistical analysis**

The receiver operating characteristic area under curve was estimated nonparametrically. The cumulative incidence of cGVHD was calculated from the date of sample draw at day 80, treating death, relapse and late acute GVHD as competing risks, and groups were compared using Gray's test. Protein concentrations between groups were compared using Wilcoxon two-sample tests. Two-sided *P* values <0.05 were considered statistically significant.

**Results**

**Discovery phase**

Two independent intact protein analyses were done with pooled plasma samples according to presence or absence of prior acute GVHD. IPA1 represents a comparison of cases and controls associated with subsequent *de novo*-onset cGVHD, and IPA2 represents a comparison associated with subsequent quiescent-onset cGVHD. Four hundred twenty-two proteins had quantitative ratios in both IPA1 and IPA2. Among them, 20 candidate biomarker proteins met either of the following criteria (Table 2): (1) concentrations were at least 1.5-fold higher or 0.66-fold lower in pooled cases compared with pooled controls in both IPA1 and IPA2 (10 proteins), or (2) concentrations were at least 1.5-fold higher or 0.66-fold lower in IPA1 but not in IPA2 (10 proteins). The second criterion was included because persistence of protein alterations associated with acute GVHD could mask the identification of protein alterations also associated with cGVHD.

Based on availability of suitable antibodies pair for ELISA measurement, 12 of the 20 identified proteins were selected for further testing in each discovery sample by ELISA (Table

2). Concentrations of these 12 proteins were measured in 38 of the 40 individual plasma samples that were used for the IPA1 and IPA2 pools. Two samples (1 case in IPA1 and 1 control in IPA2) had no left over material after IPA and were not tested by ELISA. Four proteins from IPA1 (LGALS3BP, CD5L, CD163 and TXN) and 2 proteins from IPA2 (LGALS3BP and CD5L) met the criteria for further verification (ELISA ratio consistent with mass spectrometry ratio AND area under the curve ≥0.6) (Table 3).

**Verification phase**

We then tested individual plasma samples of the 127 remaining patients who met the same eligibility criteria at day 80. Among 62 patients without prior acute GVHD, 41 (66%) developed *de novo*-onset cGVHD. The NIH global severity was mild in 4 patients (10%), moderate in 23 (56%) and severe in 14 (34%), and 33 (80%) had overlap syndrome. Among 65 patients with prior acute GVHD, 41 (63%) developed quiescent-onset cGVHD. The NIH global severity was mild in 1 patients (2%), moderate in 22 (54%) and severe in 18 (44%), and 37 (90%) had overlap syndrome. Other patient characteristics are summarized in Table 1. The cumulative incidence of subsequent cGVHD was compared according to protein concentrations at day 80, using the median concentration in the discovery cohort as the threshold (Figure 1). Among the candidate proteins, the cumulative incidence of subsequent *de novo*-onset cGVHD was higher in patients with higher plasma soluble CD163 concentrations at day 80 than those with lower concentrations (75% versus 40%, $P$=0.018). The cumulative incidence of subsequent quiescent-onset cGVHD did not differ statistically according to LGALS3BP or CD5L concentrations.

**Comparison of CD163 concentrations among patients without prior acute GVHD at day 80 with healthy individuals and with patients with active chronic GVHD**

We compared CD163 concentration among 81 combined patients without prior acute GVHD at day 80 with healthy individuals and with patients with active cGVHD at any time (Figure 2). CD163 concentrations were higher among patients with no subsequent cGVHD compared to healthy individuals ($P$=0.038), among patients with subsequent *de novo*-onset cGVHD compared to those with no subsequent cGVHD ($P$=0.039), and among patients with active cGVHD compared to those with subsequent *de novo*-onset cGVHD ($P$=0.0049).

## Discussion

In order to elucidate day 80 plasma protein profiles associated with subsequent onset of cGVHD, we used the comprehensive, reduced-bias technology of high-throughput mass spectrometry. Discovery analysis identified 20 candidate proteins. Verification analysis was carried out for 12 of these candidate proteins for which ELISA were available. Measurement of individual samples by ELISA showed that 4 of the 12 proteins held promise for further verification. Testing of independent samples by ELISA showed that higher plasma soluble CD163 concentrations at day 80 were associated with the subsequent *de novo*-onset cGVHD.

Our results suggest that monocyte/macrophage activation may contribute to the subsequent development of cGVHD. CD163 is a member of the cysteine-rich scavenger receptor superfamily and is expressed in monocytes and macrophages that have an anti-inflammatory function.[26] IL-6 and anti-inflammatory cytokines such as IL-10 increase CD163 expression in monocytes and macrophages, while pro-inflammatory mediators such as lipopolysaccharide, interferon-γ, and tumor necrosis factor-α suppress CD163 expression.[27] Classically activated macrophages demonstrate proinflammatory functions, while alternatively activated macrophages demonstrate anti-inflammatory functions, regulate tissue repair and remodeling,[28-30] mediate cGVHD in murine models,[31] and accumulate in fibrotic lesions in both mice and patients with cGVHD.[31-33] Taken together, increased concentrations of plasma CD163 could

reflect increased alternatively-activated monocytes and macrophages that are pathogenic for cGVHD.

Although CD163 concentrations at day 80 after transplantation were associated with the subsequent development of *de novo*-onset cGVHD, CD163 was not a candidate for quiescent-onset cGVHD in mass spectrometry analysis. CD163-positive macrophages in the skin and bone marrow have been associated with refractory acute GVHD and poor prognosis.[34, 35] Thus CD163 concentrations might have been strongly influenced by the prior onset of acute GVHD and its treatment, and could have obscured any association with subsequent quiescent-onset cGVHD.

CD163 also functions as an acute phase-regulated receptor involved in the clearance and endocytosis of hemoglobin-haptoglobin complexes by macrophages,[36] thereby protecting tissues from free hemoglobin-mediated oxidative damage. Expression of CD163 is constitutive and is induced by stimulation of circulating monocytes and tissue macrophages. A soluble form of CD163 is released into plasma by proteolysis after oxidative stress[37] or stimulation through toll-like receptors 2, 4 and 5.[38] The concentration of plasma soluble CD163 is elevated in oxidative conditions such as diabetes,[39] rheumatoid arthritis,[40] and systemic sclerosis.[41] Accordingly, our results suggest that higher oxidative stress may predispose toward the development of *de novo*-onset cGVHD.

Biomarker proteins associated with chronic GVHD have been examined in several studies.[12-14, 42-48] Most studies selected candidate proteins based on presumed immunological pathways or on results of studies investigating acute GVHD. One recent study identified 5 diagnostic proteins that distinguished refractory chronic GVHD patients at disease onset from those who never had GVHD. The chemokine (C-X-C motif) ligand 9 (CXCL9) was most useful for diagnosing chronic GVHD.[12] Another recent study validated a 4-protein diagnostic biomarker panel (CXCL9, ST2 [serum stimulation-2, IL1RL1 gene product], matrix metalloproteinase 3 and osteopontin) for diagnosis and severity of chronic GVHD.[13] No studies have examined

prognostic plasma biomarker proteins associated with onset of chronic GVHD by mass spectrometry.

Several studies have examined prognostic biomarker proteins associated with the subsequent onset of cGVHD based on biological interest. BAFF concentrations ≥10 ng/mL at 6 months after HCT were associated with subsequent onset of cGVHD.[14] Higher concentrations of TNF-α at 3-6 months or 2-4 weeks were associated with an increased risk of cGVHD.[45, 46] Higher concentrations of IL-10 at 4 months were associated with an increased risk of cGVHD.[45] Higher concentrations of IFN-γ at 3 months, TGF-β at 2-4 weeks and IL-15 at day 7 were associated with decreased risk of cGVHD.[45, 46, 48] None of these proteins surfaced as a candidate in our mass spectrometry analysis. This result could be explained by several possibilities: (1) different sampling time points, (2) protein abundance that was too low for mass spectrometry analysis, (3) different diagnostic criteria for cGVHD, especially considering that previous studies could have included patients with late acute GVHD, (4) different time intervals between sample collection and onset of cGVHD, and (5) differences in immunosuppressive treatment at the time of sample collection, since none of the prior studies considered prednisone doses at sample collection.

This study has several limitations. First, the study was not designed to characterize the positive-predictive value of testing CD163 concentrations as a prognostic biomarker for *de novo*-onset cGVHD. This question remains to be tested in future studies. Second, further verification of the association of CD163 concentrations with *de novo*-onset cGVHD is needed, because the current study did not account for multiple comparisons. Third, because we limited the study to patients who were on minimal doses of corticosteroids, the predictive value for patients receiving corticosteroids remains to be determined. Lastly, proteins without suitable ELISA could not be tested, and await technology advances in the future.

We have learned that the 2014 NIH consensus for biomarker development was very helpful for designing the study and for reporting the results in a standardized way.[49] This study

illustrates challenges but also the progress in design and rigor of standardization in conducting studies of biomarker development in cGVHD. Identification of prognostic biomarkers is crucial for early intervention, but it might be more challenging than identification of diagnostic biomarkers, since patients do not yet have the event at sample collection and the signals associated with the later event of interest may be subtle.

The careful design of the cohort represents a strength of our study. Since all patients had quiescent GVHD activity under minimum glucocorticoid administration at day 80, the interpretation of the results is not confounded by the possible effects of glucocorticoid administration. Although the effect of glucocorticoid administration on CD163 concentrations remains to be tested in future studies, our results suggest that that monocyte/macrophage activation or increased oxidative stress contributes to the pathogenesis of *de novo*-onset cGVHD.

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*Authorship statement:* Y.I., P.J.M. and J.A.H. designed the study. Y.I. and B.E.S. performed the statistical analysis. L.T., M.E.D.F., S.J.L., P.A.C. and J.A.H. collected samples and data. A.A.M.,

C.L.M., S.P. and S.H. assayed samples. All authors wrote and critically revised the manuscript for important intellectual content and approved the manuscript to be published.

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**Figure 1. Cumulative incidence rates of *de novo*-onset chronic GVHD according to concentrations of 4 plasma proteins at day 80 in the verification cohort.** Thresholds were derived from the median concentration in the discovery cohort.

**Figure 2. Plasma CD163 concentrations.** Samples among 81 patients without prior acute GVHD were collected at day 80, and 31 developed no chronic GVHD and 50 developed *de novo*-onset chronic GVHD. Samples of active chronic GVHD were collected at any time. Values are represented by dot blots with the mean ± standard error of mean. *P* values are derived from Wilcoxon two-sample tests.

**Table 1. Patient characteristics**

| Characteristic, no. (%) | Discovery cohort (n=40) | | | | Verification cohort (n=127) | |
|---|---|---|---|---|---|---|
| | IPA 1: Prior acute GVHD – Subsequent chronic GVHD | | IPA 2: Prior acute GVHD + Subsequent chronic GVHD | | Subsequent chronic GVHD | |
| | Yes (n=10) | No (n=10) | Yes (n=10) | No (n=10) | Yes (n=82) | No (n=45) |
| **Prior acute GVHD** | 0 (0) | 0 (0) | 10 (100) | 10 (100) | 41 (50) | 24 (53) |
| **Median patient age at transplantation (range)** | 54 (36-63) | 43 (32-59) | 48 (28-71) | 48 (19-64) | 55 (26-73) | 52 (20-69) |
| **Median months from sample collection to chronic GVHD** | 2.7 (1.4-5.0) | NA | 5.2 (0.5-16) | NA | 4.9 (0.5-31) | NA |
| **Patient gender** | | | | | | |
| Male | 5 (50) | 5 (50) | 5 (50) | 5 (50) | 58 (71) | 25 (56) |
| Female | 5 (50) | 5 (50) | 5 (50) | 5 (50) | 24 (29) | 20 (44) |
| **Disease risk at transplantation*** | | | | | | |
| Standard | 5 (50) | 5 (50) | 3 (30) | 5 (50) | 27 (33) | 17 (38) |
| High | 5 (50) | 5 (50) | 7 (70) | 5 (50) | 55 (67) | 28 (62) |
| **HLA and donor type** | | | | | | |
| Matched related | 7 (70) | 5 (50) | 6 (60) | 5 (50) | 33 (40) | 19 (42) |
| Matched unrelated | 2 (20) | 3 (30) | 4 (40) | 3 (30) | 36 (44) | 17 (38) |
| HLA mismatched | 1 (10) | 2 (20) | 0 (0) | 2 (20) | 13 (16) | 9 (20) |
| **Conditioning regimen** | | | | | | |
| High intensity | 4 (40) | 7 (70) | 5 (50) | 8 (80) | 45 (55) | 21 (47) |
| Reduced intensity | 6 (60) | 3 (30) | 5 (50) | 2 (20) | 37 (45) | 24 (53) |
| **Graft source** | | | | | | |
| Bone marrow | 0 (0) | 2 (20) | 0 (0) | 4 (40) | 6 (7) | 11 (24) |
| Mobilized blood cells | 10 (100) | 8 (80) | 10 (100) | 6 (60) | 76 (93) | 32 (71) |
| Umbilical cord blood | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (4) |
| **T cell depletion** | | | | | | |
| Antithymocyte globulin | 0 (0) | 1 (10) | 0 (0) | 2 (20) | 1 (1) | 1 (2) |
| Campath | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

| **Immunosuppressive drugs at sample collection** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Calcineurin inhibitor | 10 (100) | 10 (100) | | 8 (80) | 10 (100) | | 80 (98) | 41 (91) |
| Mycophenolate mofetil | 1 (10) | 0 (0) | | 2 (20) | 0 (0) | | 21 (26) | 12 (27) |
| Sirolimus | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | | 3 (4) | 1 (2) |

IPA indicates intact protein analysis; GVHD, graft-versus-host disease; and HLA, human leukocyte antigen.
*The standard-risk category included chronic myeloid leukemia in chronic phase, acute leukemia in first remission, myelodysplastic syndrome without excess blasts, and non-malignant diseases. The high-risk category included all other diseases and stages.

**Table 2. Candidate biomarker proteins associated with development of chronic GVHD**

| Protein | Gene | Gene description | IPA1: *De novo* chronic GVHD | | IPA2: Quiescent chronic GVHD | | Suitable ELISA Antibodies Pair |
|---|---|---|---|---|---|---|---|
| | | | Ratio | #Events | Ratio | #Events | |
| SCAND3 | *SCND* | SCAN domain containing 3 | 13.6 | 9 | 21.4 | 6 | No |
| KCNH | *KCNH* | potassium voltage-gated channel, subfamily H (eag-related) | 13.4 | 3 | 16.9 | 6 | No |
| KIAA1958 | *K1958* | KIAA1958 | 18.5 | 2 | 16.3 | 3 | No |
| **IGHM** | *IGHM* | immunoglobulin heavy constant mu | 4.5 | 78 | 6.3 | 7 | **Yes** |
| **HPR** | *HPTR* | haptoglobin-related protein | 2.4 | 261 | 2.6 | 126 | **Yes** |
| **LGALS3BP** | *LG3BP* | lectin, galactoside-binding, soluble, 3 binding protein | 1.9 | 71 | 2.5 | 52 | **Yes** |
| **GIP** | *GIP* | gastric inhibitory polypeptide | 2.1 | 5 | 2.1 | 6 | **Yes** |
| **CD5L** | *CD5L* | CD5 molecule-like | 2.2 | 174 | 2.1 | 45 | **Yes** |
| **LTBP2** | *LTBP2* | latent transforming growth factor beta binding protein 2 | 1.6 | 7 | 1.6 | 3 | **Yes** |
| SH3BGRL3 | *SH3L3* | SH3 domain binding glutamic acid-rich protein like 3 | 0.6 | 6 | 0.65 | 8 | No |
| LILRA3 | *LIRA3* | leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3 | 1.5 | 39 | 1.3 | 40 | No |
| **CD163** | *C163A* | CD163 molecule | 1.7 | 29 | 1.3 | 14 | **Yes** |
| GUCA2A | *GUC2A* | guanylate cyclase activator 2A (guanylin) | 1.6 | 5 | 1.3 | 9 | No |
| **CRTAC1** | *CRAC1* | cartilage acidic protein 1 | 1.6 | 50 | 1.1 | 49 | **Yes** |
| PROZ | *PROZ* | protein Z, vitamin K-dependent plasma glycoprotein | 1.5 | 114 | 1.1 | 64 | No |
| PRDX6 | *PRDX6* | peroxiredoxin 6 | 1.7 | 17 | 0.70 | 7 | No |
| **TFF2** | *TFF2* | trefoil factor 2 | 1.7 | 2 | 0.68 | 2 | **Yes** |
| **TXN** | *THIO* | thioredoxin | 1.7 | 18 | 0.67 | 28 | **Yes** |
| **HSP90B1** | *ENPL* | heat shock protein 90kDa beta (Grp94), member 1 | 0.6 | 19 | 1.3 | 15 | **Yes** |
| **PDLIM1** | *PDLI1* | PDZ and LIM domain 1 | 0.6 | 32 | 0.73 | 19 | **Yes** |

**Table 3. Candidate selection comparing cases with controls in the discovery phase**

| Protein | IPA1: *De novo* chronic GVHD | | | | | | IPA2: Quiescent chronic GVHD | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MS Ratio | ELISA Ratio | Ratio Consistency | AUC | Candidate | | MS Ratio | ELISA Ratio | Ratio Consistency | AUC | Candidate |
| IGHM | 4.5 | 1.3 | Yes | 0.58 | No | | 6.31 | 1.4 | **Yes** | 0.51 | No |
| HPR | 2.4 | 0.65 | No | – | No | | 2.59 | 0.86 | No | – | No |
| LGALS3BP | 1.9 | 1.43 | Yes | **0.68** | **Yes** | | 2.45 | 1.59 | **Yes** | **0.64** | **Yes** |
| GIP | 2.1 | 1.00 | No | – | No | | 2.11 | 0.63 | No | – | No |
| CD5L | 2.2 | 1.83 | Yes | **0.66** | **Yes** | | 2.1 | 1.63 | **Yes** | **0.66** | **Yes** |
| LTBP2 | 1.6 | 0.85 | No | – | No | | 1.63 | 0.93 | No | – | No |
| CD163 | 1.7 | 1.34 | Yes | **0.73** | **Yes** | | | | | | |
| CRTAC1 | 1.6 | NA | No | – | No | | | | | | |
| TFF2 | 1.7 | 0.94 | No | – | No | | | | | | |
| TXN | 1.7 | 2.13 | Yes | **0.70** | **Yes** | | | | | | |
| HSP90B1 | 0.6 | 2 | No | – | No | | | | | | |
| PDLIM1 | 0.6 | 3.11 | No | – | No | | | | | | |

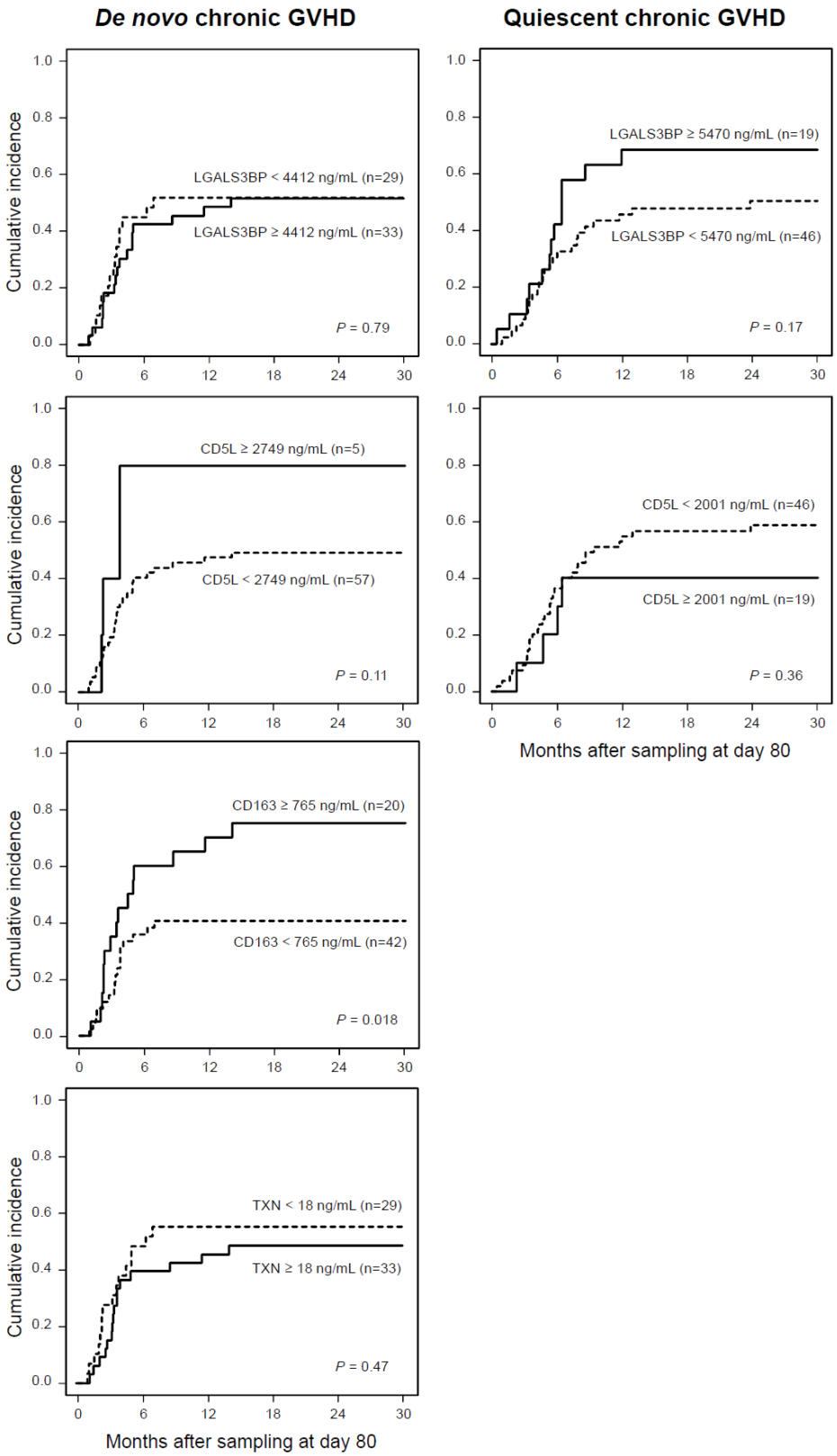


**Figure 1**

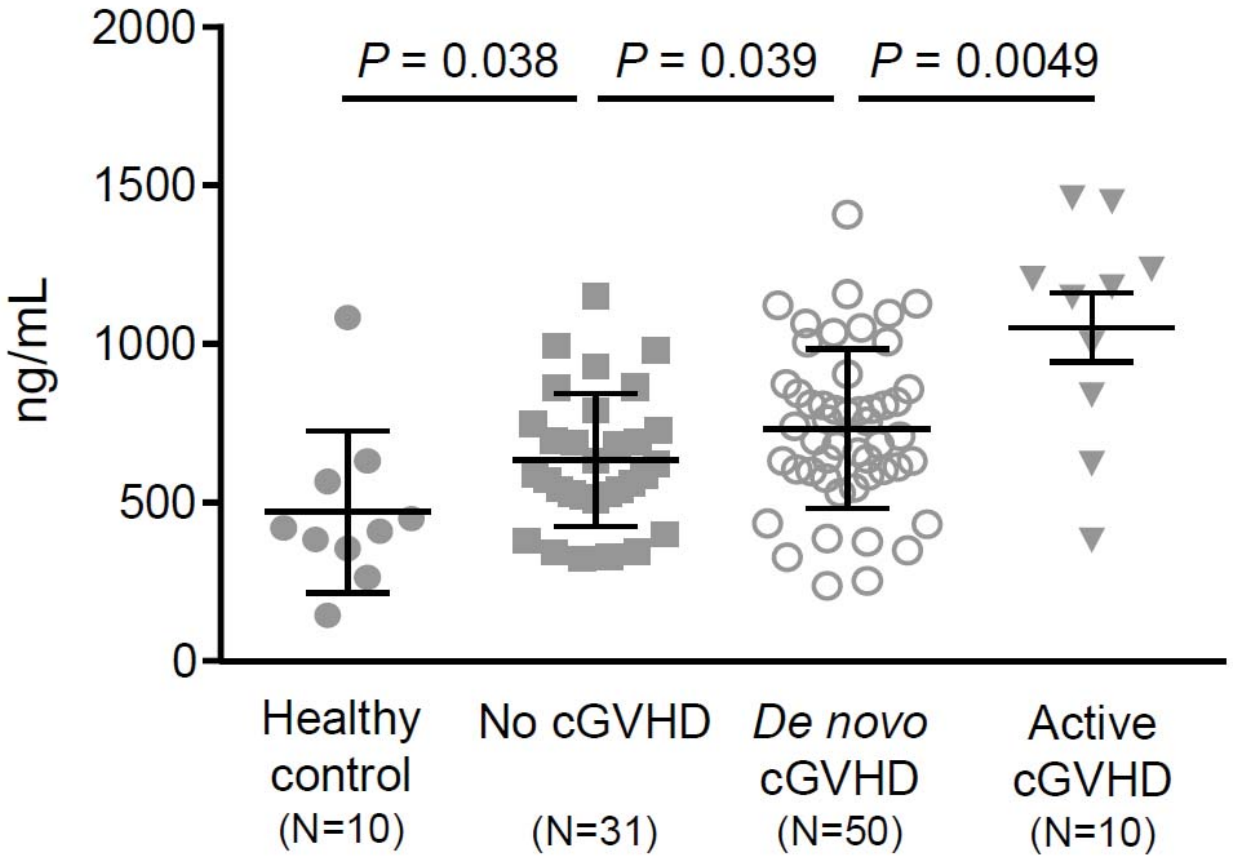


**Figure 2**